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PRINCIPAL INVESTIGATOR: Lewis G. Sheffield, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, WI 53706

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FOREWORD

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INTRODUCTION

The hypothesis of this proposal is that ErbB2 expression regulates the activity of c-src and that this activation is a factor in mediating ErbB2 induced tumorigenesis. Previous work with human tumors has shown increased c-src activity (Ottenhoff-Klaff et al., 1992). Tissue from mice overexpressing ErbB2 show increased activity of c-src (Muthuswamy et al., 1994). Knockout mice lacking c-src also exhibit decreased tumorigenesis induced by polyomavirus middle T antigen (Guy et al., 1994). Data from our laboratory and others (Zhair et al., 1993) show that non-tumorigenic mammary epithelial cells are readily transformed to a tumorigenic phenotype by overexpression of ErbB2. This tumorigenic transformation is accompanied by increased activity of c-src (see below).

The overall project has two major areas of focus: the mechanism by which ErbB2 activates c-src and the consequences of c-src activation. Earlier work focused on the activation of c-src by ErbB2. There are several ways by which ErbB2 could regulate c-src activity: by altering c-src expression levels, by decreasing expression and/or activity of csk, by increasing expression and/or activity of c-src-directed phosphotyrosine phosphatases or by phosphorylation-independent activation of c-src. These possible mechanisms were addressed in Year 2 work.

As described in the STATEMENT OF WORK, year 3 consisted of further assessing effects of ErbB2 on c-src phosphatases and further defining the impact of inhibiting src activation (through dominant negative inhibitors and SHP2 inhibitors) on the ErbB2-transformed phenotype.

BODY

Experimental Methods

Cell Lines

Parental Lines

The nontumorigenic human mammary epithelial cell line 184.A1 and the nontumorigenic mouse line NMuMG was used in most studies covered by this report. Cells were routinely grown in DMEM:F12 + 10% FBS, 5 µg/ml insulin and 10 ng/ml EGF.

Transfected lines

Cells were transfected by ErbB2 as previously described (Sheffield, 1998). For studies on the functional consequences of c-src activation by ErbB2, cells were initially transfected with dominant negative c-src or dominant negative SHP2 under control of the CMV promoter and in a neomycin resistance vector (Bell et al., 1992) or with empty vector. These cells were then selected for stable transfection by selection in media containing G418 to give control and dominant negative c-src transformed cells. The ErbB2 was then excised from the initial neomycin resistance vector and subcloned into a Zeocin resistance vector. Control and dominant negative c-src cells (above) were then transfected with this vector or with the empty vector and selected in media containing G418 and Zeocin.

Measurement of phosphatase activity

Cells were lysed with 50 mM HEPES containing 40 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM Na^3VO_4 , 1 mM PMSF and 1% Triton X-100. Protein content was determined by BCA method (Pierce Chemical Co., Rockford, IL), equalized among samples and ErbB2 immunoprecipitated using anti-ErbB2 and agarose conjugated Protein A/G. Phosphatase activity assessed by the Malacite Green method of assessing free phosphate (Harder et al., 1994) and using a peptide corresponding to the C-terminus of src (peptide 301, Biomole, Plymouth Meeting, PA) or a peptide corresponding to the autophosphorylation site of src (peptide 312, Biomole) as substrate peptides.

Western Analysis of phosphatase activity

Cell lysates, membranes or ErbB2 immunoprecipitates were prepared as described above. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and western blots probed with antibodies to the indicated phosphatases. Computer densitometry was used to assess relative band intensity.

Results***Summary of Year 1 and 2 Results***

For background and to place Year 3 results in context, Year 1 and 2 results are summarized briefly. Data supporting these conclusions were reported in year 1 Progress Report.

Year 1

1. ErbB2 vector efficiently increased expression of ErbB2 in mammary epithelial cells
2. ErbB2 transfection leads to a transformed phenotype, as assessed by growth on soft agar and in athymic mice
3. ErbB2 transformation leads to increased activity of c-src without increasing expression of c-src.
4. The c-src assay was linear over time and amount of enzyme, was dependent on added substrate and conducted under substrate and ATP concentrations leading to maximum activity. Results did not appear to be due to suboptimum assay conditions.
5. C-src phosphorylation pattern in ErbB2 transformed cells was consistent with dephosphorylation at Y527 in response to ErbB2.
6. These results appeared to be reproducible in other mammary epithelial cell lines.

Year 2

7. ErbB2 increased the content and activity of CSK in mammary epithelial cells
8. ErbB2 increased the activity of phosphatases directed toward a peptide corresponding to the C-terminus of c-src.
9. A large portion of the src-directed phosphatase activity co-immunoprecipitated with ErbB2.
10. SHP2 was tentatively identified as an ErbB2-associated phosphatase.

11. Co-transfection of ErbB2 and a dominant negative c-src decreased soft agar growth of mammary epithelial cells

Association of ErbB2 with SHP2

Co-immunoprecipitation studies, followed by western blot analysis, indicated that a major phosphatase associated with ErbB2 was SHP2 (Figure 1). SHP2 precipitated with ErbB2 appeared to largely parallel the amount of ErbB2 in samples, and the phosphatase activity directed toward the c-terminus of c-src.

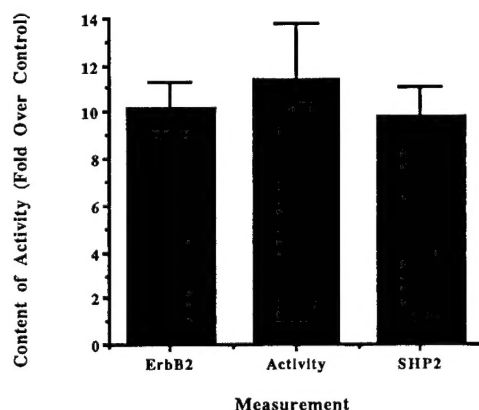


Figure 1. Effect of ErbB2 on ErbB2-associated phosphatase. ErbB2 was immunoprecipitated from wild-type (Control) or ErbB2-transformed cells. ErbB2 and SHP2 content were determined by western blot analysis. Phosphatase activity was determined by de-phosphorylation of peptide corresponding to the C-terminus of c-src as described in Methods. Data expressed relative to wild-type (Control) cells. Mean \pm SEM of 5 determinations.

Co-transfection of ErbB2 and dominant negative SHP2

To further assess the role of SHP2 in ErbB2-induced activation of c-src, cells were co-transfected with ErbB2 and vector alone (control) or ErbB2 and dominant negative SHP2 (a truncation mutation lacking the phosphatase domain of SHP2). Expression of dominant negative SHP2 dramatically reduced the association of phosphatase activity with ErbB2 (Figure 2), suggesting that a major phosphatase associated with ErbB2 is SHP2. In additional studies, the impact of dominant negative SHP2 on c-src activation by ErbB2 was assessed. Expression of

inactive SHP2 prevented the ErbB2-induced activation of c-src (Figure 3). Thus, a major pathway for ErbB2-activation of c-src appears to be activation of SHP2.

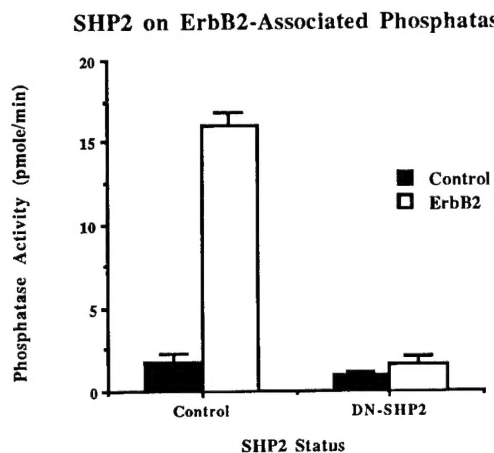


Figure 2. Effect of dominant negative SHP2 on ErbB2-associated phosphatase activity. Cells transfected with vector alone (Control) or dominant negative SHP2 (DN-SHP2) were transfected with vector (Control) or ErbB2. ErbB2 was immunoprecipitated and phosphatase activity determined as dephosphorylation of peptide corresponding to the C-terminus of C-src. Mean \pm SEM of 4 experiments.

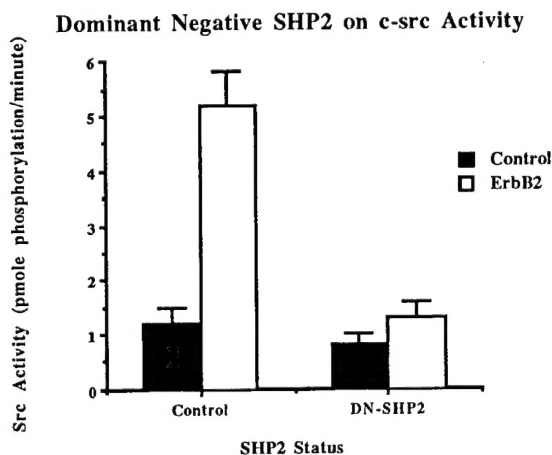


Figure 3. Effect of dominant negative SHP2 on ErbB2-induced c-src activity. Cells transfected with vector alone (Control) or dominant negative SHP2 (DN-SHP2) were transfected with vector (Control) or ErbB2. C-src activity was determined as described previously (Sheffield, 1998). Mean \pm SEM of 4 experiments.

Dominant negative src alters tumor formation

Previously, dominant negative c-src was found to inhibit the ability of mammary epithelial cells to grow on soft agar. As an additional test of tumor formation, cells were xenografted to athymic (nude) mice. After 2 months, 12/12 control tumors (ErbB2 and vector alone) were detectable, whereas only 7/12 ErbB2-dominant negative src injections formed tumors. Furthermore, the growth rate of the control cells was significantly faster, resulting in larger final tumor weight (1.2 ± 0.3 g in controls vs 0.4 ± 0.2 g in dominant negative src expressing cells). These results indicate that c-src activation by ErbB2 appears to be necessary for tumor formation and development.

Discussion

Previous results (detailed above and in Year 1 and 2 progress report) indicated that c-src was activated by ErbB2 without increasing c-src expression. Standard assay validations indicated that this was due to a true increase in src activity. A major objective of the overall project is to determine how ErbB2 activates c-src. This activation is associated with decreased phosphorylation at Y527. Presumably, the decreased Y527 phosphorylation could be due to decreased CSK activity or increased phosphatase activity. These studies indicate that decreased CSK activity does not appear to explain src activation by ErbB2. However, increased activity of SHP2 appears to be a plausible mechanism by which ErbB2 could activate src. In the present study, we determined that SHP2 was associated with ErbB2. This association does not appear to be modified by tumorigenic transformation, as the increase in SHP2 associated with ErbB2 immunoprecipitates was largely parallel with increased ErbB2 content of cells. In addition, phosphatase inactive SHP2 decreased ErbB2-induced phosphatase activity and the ability of ErbB2 to activate c-src.

In studies of the impact of c-src activation, we have shown that c-src appears to be necessary for growth of cells in soft agar and for tumor formation in athymic mice. These studies suggest that the activation of SHP2 by association with ErbB2 may be a major event in tumorigenesis, possibly by SHP2-mediated activation of c-src.

Recommendations

Based on the results of Years 1-3, the plan of work outlined for year 4 remains plausible. This includes further examination of the phenotype of cells co-transfected with dominant negative c-src and dominant negative SHP2, including formation of metastatic tumors and the ability of cells to invade extracellular matrices.

KEY RESEARCH ACCOMPLISHMENTS

- ErbB2 transformation of mammary epithelial cells leads to increased activity of c-src without increasing expression of c-src.
- C-src phosphorylation pattern in ErbB2 transformed cells was consistent with dephosphorylation at Y527 in response to ErbB2.
- c-src dephosphorylation on Y527 does not appear to be due to decreased CSK content or activity.
- c-src dephosphorylation in response to ErbB2 appears to be mediated by increased SHP2 activity.
- c-src activation by ErbB2 appears to be necessary for ErbB2-induced tumorigenicity.

REPORTABLE OUTCOMES

Manuscripts

Sheffield, L.G. 1998. Role of c-src activation in ErbB2-induced transformation of human breast epithelium. *Biochem. Biophys. Res. Commun.* 250:27-31.

Sheffield, L.G., Smuga-Otto, K., Lewandowski, J.A., Vilhubner, K. ErbB2 activates of c-src via the phosphotyrosine phosphatase SHP2. Manuscript in preparation.

CONCLUSIONS

Previous results indicate that ErbB2 increases activity of c-src in mammary epithelium. The present studies suggest that this induction of c-src activity is mediated by increased activity of a src-directed kinase that removes an inhibitory phosphate at Y527. SHP2 appears to be a leading candidate for the phosphatase. In addition, the activation of c-src by ErbB2 appears to play a

critical role in inducing a tumor phenotype. These results that strategies to modify src activity or the activity of the src-directed phosphatase(s) may prove useful in modifying tumor progression.

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C-Src Activation by ErbB2 Leads to Attachment-Independent Growth of Human Breast Epithelial Cells

Lewis G. Sheffield

Endocrinology-Reproductive Physiology Program, University of Wisconsin, Madison, Wisconsin 53706

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Nontumorigenic human mammary epithelial cells (184.A1 line) were stably transfected with ErbB2 or with Ha-Ras. Transformation with ErbB2, but not ras, resulted in a 5-6 fold increase in c-src activity without affecting c-src content of cells. Similar activation of c-src by ErbB2 was also observed in other non-tumorigenic mammary epithelial cells, including the human line MCF10A and the mouse line NMuMG. Activation of c-src appeared to be dependent on active ErbB2 tyrosine kinase, as the ErbB2 inhibitor tyrphostin AG 825 blocked the induction of c-src kinase activity, as well as the ability of transformed cells to grow on soft agar, but not plastic. The src-selective inhibitor PP1 effectively reduced c-src activity, as well as growth of ErbB2-transformed cells on soft agar, but not on plastic. These results indicate that activation of c-src is a consequence of ErbB2 kinase activity in human breast cancer cells overexpressing ErbB2, and that increased activity of c-src may be responsible for attachment-independent growth of the cells. © 1998 Academic Press

ErbB2 (neu) is overexpressed in 10-30% of human breast cancers (1). ErbB2 expression has been associated with aggressively growing tumors and poor prognosis (2, 3). Such effects are thought to be a result of ErbB2 activity, rather than correlatory effect, since ErbB2 expression in rodent mammary tissue by either in situ gene transfer or transgenic technology results in the development of mammary carcinoma at young ages (4-7). Furthermore, nontumorigenic human mammary epithelial cells can be tumorigenically transformed by expression of ErbB2 (8, 9).

The product of the ErbB2 gene is a tyrosine kinase in the same family as EGF receptor, but with no known ligand (10). A variety of signal transduction events mediated by ErbB2 have been described, including activation of ras-MAPK and PI_3 Kinase pathways (11, 12). In mice transgenic for ErbB2, tumor tissue contains elevated c-src activity relative to non-tumor tissue (13).

These results suggest that ErbB2 overexpression causes an activation of c-src, which may be at least partly responsible for the phenotype of ErbB2 transformed cells. Therefore, the objective of this study was to determine if overexpressing ErbB2 results in activation of c-src in non-tumorigenic human mammary epithelial cells and if c-src activation may be responsible for tumor phenotype.

MATERIALS AND METHODS

Cell lines. 184.A1 human mammary epithelial cells (14), MCF10A human mammary epithelial cells (15) and NMuMG mouse mammary epithelial cells (16) were obtained from ATCC (Rockville, MD). Cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 ng/ml EGF. pJRneu (17) and pJRras (18) were kindly provided by Dr. M. Gould (University of Wisconsin, Madison). pJRneu is based on the plasmid pJR containing tumorigenic human neu, driven by CMV promoter and containing a neomycin resistance marker. pJRras is similar, except that v-Ha-ras is present instead of neu. Transfected cell lines were selected in media containing 400 μ g/ml G418 sulfate.

Western blot analysis. To assess expression of ErbB2 and c-src, cells were grown to near confluency and lysed with SDS loading buffer (19) lacking 2-mercaptoethanol and bromophenol blue. Protein content of samples was determined by BCA assay (Pierce Chemical Co., Rockford, IL), 2-mercaptoethanol and bromophenol blue added to the samples and 50 μ g protein separated by SDS-PAGE. Proteins were transferred to PVDF membranes and western blots probed with antibodies against c-src (UBI, Lake Placid, NY) essentially as described by Fenton and Sheffield (20).

c-src activity. Cells grown to approximately 80% confluency were lysed with lysis buffer (50 mM HEPES, pH 7.0 containing 30 mM sodium pyrophosphate, 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% BSA, 1 mM sodium orthovanadate and 1 mM PMSF) and centrifuged (15,000 g for 15 minutes). Protein content of the supernatant was determined by BCA assay, equalized among cell lines and c-src immunoprecipitated from equal protein amounts essentially as previously described (21) using anti-c-src (UBI) and agarose conjugated protein A and G (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed 4 times with lysis buffer and c-src activity determined by incubating immunoprecipitated enzyme in 15 μ l of assay buffer (200 mM HEPES, pH 7.0 containing 125 mM $MgCl_2$, 25 mM $MnCl_2$, and 0.25 mM sodium orthovanadate) with or without substrate peptide ([lys¹⁹]cdc2(6-20)) or control pep-

tide ([phe¹⁵lys¹⁹]cdc2(6-20)) (1 mM for standard assays). Reactions were started by adding 5 μ M of a 0.5 mM γ -³²P-ATP solution (approximately 1 Ci/mmol, Dupont, Boston, MA). Standard reactions were continued for 5 minutes and stopped by adding 10% trichloroacetic acid and 100 μ g bovine serum albumin. Samples were centrifuged (3000 g for 5 minutes) and supernatant spotted onto Whatman P81 phosphocellulose paper. Paper was washed 5 times with 100 mM phosphoric acid, dried and counted by liquid scintillation. c-src content in immunoprecipitates was determined by western blot analysis as described above, quantitated by computer-assisted densitometry (Collage, Fotodyne, New Berlin, WI) and activity adjusted for c-src content.

Cell growth. Control or ErbB2 transfected cells were plated onto petri dishes (10⁴ cells/cm²), treated as described and allowed to grow for 3 days with media changed each day. Cells were counted by hemocytometer counting and population doubling time estimated as an index of growth rate.

Soft agar growth. Wild-type or ErbB2 transformed cells were suspended in DMEM containing 10% fetal bovine serum and 0.3 % agar

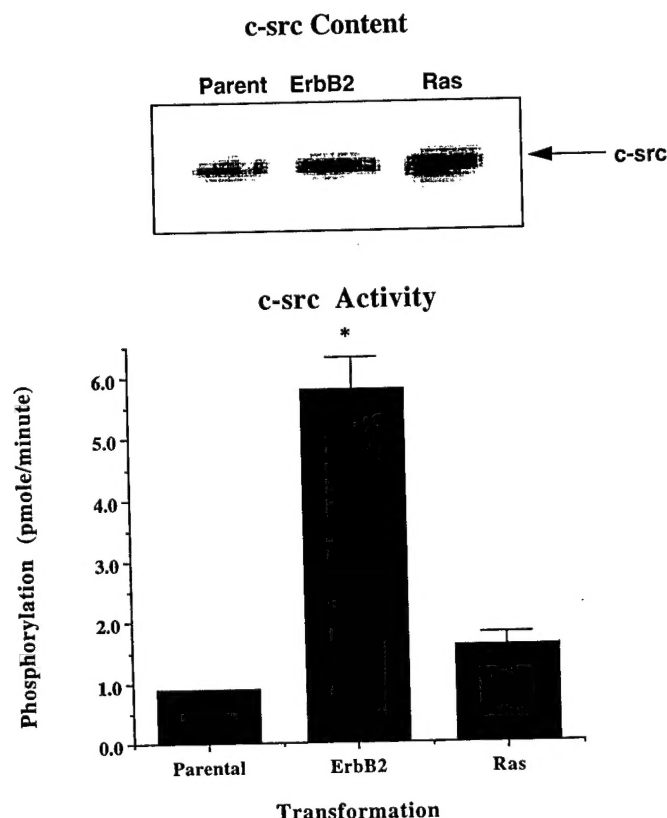


FIG. 1. c-src content and activity of parental, ErbB2 transformed and ras-transformed 184.A1 human mammary epithelial cells. For both studies, parental, ErbB2 and ras transformed cells were generated and cultured as described in Materials and Methods. A. Cells were lysed with SDS loading buffer, proteins separated by SDS PAGE and western blot analysis was performed as described in Materials and Methods. Representative of 3 experiments. Densitometry analysis indicated no significant differences in c-src expression. B. c-src was immunoprecipitated, c-src content of immunoprecipitates assessed by western analysis and kinase activity determined as described in Materials and Methods. c-src activity was reported prior to normalization to c-src content of immunoprecipitates. Mean \pm SEM of 3 experiments. * = Significantly different than parental line, $P < 0.05$.

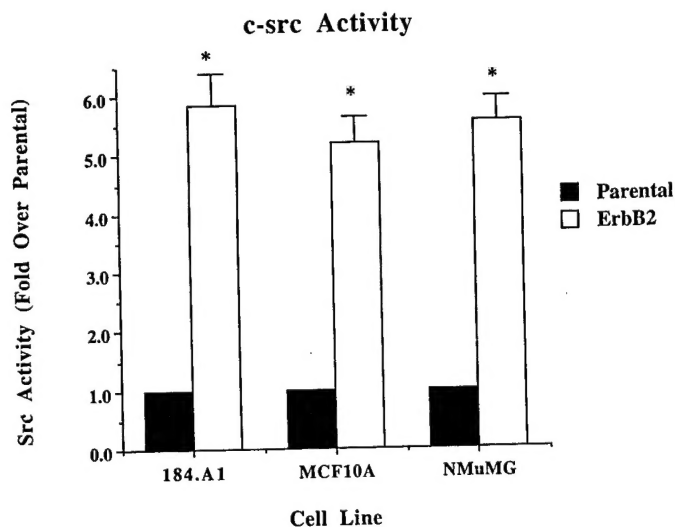


FIG. 2. Comparison of cell lines. Parental, ErbB2 and ras transformed 184.A1, MCF-10A and NMuMG cells were generated as described in Materials and Methods. c-src was immunoprecipitated, c-src content of immunoprecipitates determined by western blot analysis and kinase activity of immunoprecipitates determined as described in Materials and Methods. Kinase activity was reported after normalization to c-src content of immunoprecipitates. Mean \pm SEM of 3 determinations. * = Significantly different than parental line, $P < 0.05$.

and plated over 0.5 % bottom agar at a density of 10³ cells/100 mm petri dish. Cells were then treated as described and colony forming efficiency determined after 7 days of growth.

Statistical analysis. All experiments were replicated on at least 3 occasions. Quantitative data were analyzed by analysis of variance (ANOVA) and transformed cell lines compared with parental cells by Dunnett's t-test (22). Unless otherwise states, all differences noted were $P < 0.05$.

RESULTS

The c-src content of parental, ErbB2 transformed and ras transformed cells did not vary significantly among the 3 lines (Figure 1A). Despite similar levels of c-src, phosphorylation of a c-src substrate peptide was increased approximately 5-6 fold in ErbB2 expressing cells compared with control cells (Figure 1B). The c-src activity in ras-transformed cells was not significantly different than parental cells, suggesting that this effect was not a general effect of transformation but was likely due to ErbB2 expression. In preliminary studies (not shown), c-src kinase activity was found to be dependent on the presence of substrate peptide, linear over time and linear over the amount of cell extract used for immunoprecipitation.

The effect of ErbB2 transformation on c-src activity was not confined to 184.A1 cells. The nontumorigenic human mammary epithelial cell line MCF-10A and the mouse line NMuMG also exhibited substantial increases in c-src activity upon transfection with ErbB2, but not with ras (Figure 2).

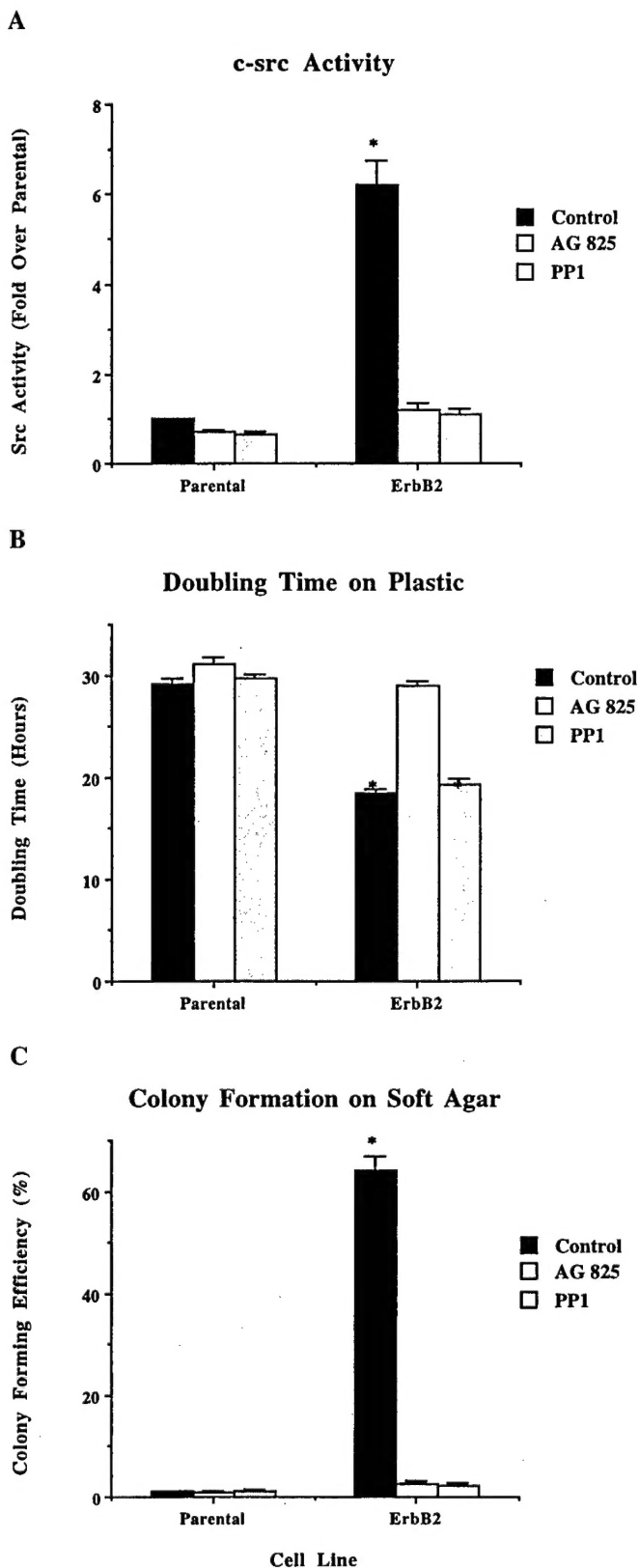


FIG. 3. Influence of Tyrphostin AG 825 (ErbB2 inhibitor) or PP1 (src inhibitor) on c-src activity (A), growth on plastic (B) and growth on soft agar (C). Parental and ErbB2 transformed 184.A1 cells were generated as described in Materials and Methods, cultured on plastic

In order to further verify that the observed change in c-src activity was due to ErbB2 activity, the effects of the selective ErbB2 inhibitor Tyrphostin AG 825 on c-src activation, cell growth and attachment independent growth was assessed. AG 825 (10 μ M) dramatically decreased c-src activity in ErbB2 transformed cells, to levels approximately equal to control levels (Figure 3A). This concentration had no effect on cell viability, as measured by trypan blue exclusion (not shown). Cell proliferation on plastic was slightly reduced by AG 825. Untreated ErbB2 transformed cells grew significantly faster than parental cells. However, AG 825 reduced the growth rate of ErbB2 transformed cells to rates that were not different than parental cells (Figure 3B). Parental cells were largely incapable of forming colonies on soft agar, while ErbB2 transformed cells readily formed colonies on soft agar. AG 825 dramatically inhibited the ability of ErbB2 transformed cells to form colonies on soft agar (Figure 3C).

In order to evaluate possible roles of c-src in the ErbB2 tumor phenotype, the ability of the putative c-src inhibitor PP1 to reverse tumor phenotype was examined. PP1 reduced c-src activity in ErbB2 transformed cells (Figure 3A). At a dose that resulted in c-src activity similar to control levels (100 nM), PP1 had little effect on cell proliferation on plastic and ErbB2 transformed cells continued to grow faster than parental cells (Figure 3B). However, PP1 dramatically inhibited growth of ErbB2 transformed cells on soft agar (Figure 3C). These results suggest that c-src activation by ErbB2 may have little effect on mitogenesis of human breast cancer, but may play an important role in other tumor phenotypes, such as attachment independent growth.

DISCUSSION

The present study indicates that overexpression of ErbB2 in a non-tumorigenic human mammary epithelial cell line, which has previously been shown to result in tumorigenic transformation of the cells (9), results in dramatically increased c-src activity, in the absence of altered c-src content. These results indicate that previously observed associations between ErbB2 expression and c-src activity (23-25) are likely to be a result of ErbB2 expression, and not a coincidental correlation.

(A and B) or soft agar (C) and treated with Tyrphostin AG 825 (10 μ M, AG) or PP1 (100 nM). A. c-src was immunoprecipitated, c-src content determined by western blot analysis, kinase activity determined as described in Materials and Methods and kinase activity normalized to c-src content of immunoprecipitates. B. Cells were plated, cultured for 3 days and growth rate was expressed as population doubling time. C. Cells were plated onto soft agar, cultured for 7 days and colony forming efficiency was estimated. Mean \pm SEM of 4 experiments. * = Significantly different than control treated parental cells, $P < 0.05$.

Furthermore, the results indicate that previously reported increases in c-src activity in transgenic mice expressing ErbB2 in mammary tissue (13) is also observed in human mammary epithelium. The lack of effect of ras transformation suggests that the observed effect is not a general result of transfection, selection or transformation, but is likely due to specific signaling pathways.

Because ErbB2 is known to function as a tyrosine kinase (10), we also examined the effects of the ErbB2 selective kinase inhibitor Tyrphostin AG 825 on c-src activity. This inhibitor appears to have substantial selectivity for ErbB2 over other tyrosine kinases, including c-src and ErbB1 (26). However, as with all inhibitor studies, possible non-specific effects cannot be completely eliminated. Nonetheless, these studies suggest ErbB2-mediated c-src activation is likely to depend on the tyrosine kinase activity of ErbB2. However, the exact pathway involved is not elucidated in these studies. Presumably, the pathway could involve activation of phosphotyrosine phosphatases (9, 27), inhibition of CSK-like activities (28) or direct association with ErbB2 (29).

Functionally, c-src activation could mediate a variety of tumorous phenotypes. Guy et al. (30) observed that c-src ablation decreased Polyoma virus middle-T antigen-induced mammary tumorigenesis in transgenic mice. Other researchers have reported that c-src mediated signaling may be important in cell cycle progression (31). In addition, src is well known as a mediator of cytoskeletal architecture, cell adhesion and motility (32, 33). Such processes are clearly implicated in tumor metastasis (34). Members of the c-src family have also been associated with integrin signaling complexes, which may be involved in tumor development and metastasis (35). Since ErbB2 is associated with increased tumor invasiveness and poor prognosis, the hypothesis that c-src activation by ErbB2 contributes to the highly invasive phenotype of ErbB2 expressing tumors is attractive, but as yet unproven.

In order to evaluate the possible role of c-src in tumor development, the src family selective inhibitor PP1 was used. Originally, PP1 was described as an inhibitor of Lck and Fyn (36), but is also capable of inhibiting c-src (37). In contrast to recent studies on ErbB2-induced mouse mammary tumors, which found that PP1 did not affect attachment independent cell growth (38), the present study found that proliferation of ErbB2-transformed human mammary epithelium on plastic was unaffected by PP1, but soft agar growth was dramatically inhibited by PP1. These results suggest that c-src activation by ErbB2 may not be important in mediating cell cycle progression, but may be critical for mediating other aspects of the transformed phenotype.

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